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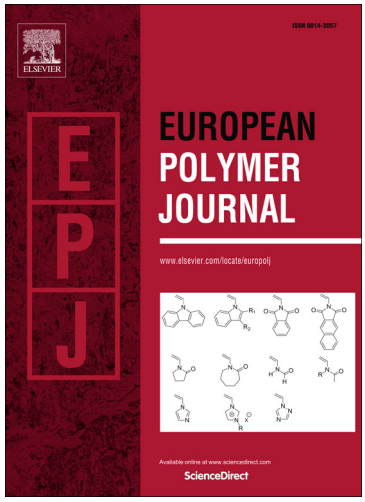


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# A direct comparison of linear and star-shaped poly(dimethylaminoethyl acrylate) polymers for polyplexation with DNA and cytotoxicity in cultured cell lines

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**Abstract**

Poly[2-(Dimethylamino) ethyl acrylate] (PDMAEA) based polymers have been studied as potential gene delivery system. However, few reports emerging in literature suggesting that star-shaped PDMAEA based polymers are performing better in polyplexation with DNA, cytotoxicity and transfection, as compared to linear counterparts. Nonetheless, little evidences exist on direct comparison between the linear and star-shaped polymer structures. To address this, a series of new star-shaped PDMAEA polymers with linear counterparts were synthesised and directly compared their polyplexation with DNA and cytotoxicity in culture cell lines. The star-shaped PDMAEA polymers were synthesised using pentaerythritol tetrakis [2-(dodecylthiocarbonothioylthio)-2-methylpropionate] (4-arm DDMAT) RAFT agent in a "core-first" approach, whereas 2-(dodecylthiocarbonothioylthio)-2-methylpropionate was used to synthesise linear PDMAEA polymers. In order to investigate the effect of molar mass, both star-shaped and linear PDMAEA were synthesised in low (10kDa) and high (20kDa) molar mass. It must be noted here that the overall molar mass of the star-shaped polymer was equal to that of the linear counterparts. Interestingly, we found that the star-shaped polymer has slightly smaller hydrodynamic diameter (more compact) relative to linear counterparts, and importantly, star-shaped PDMAEA binds to DNA at much lower nitrogen to phosphate ratio (N/P ratio). However, the cytotoxicity studies in cultured 3T3 murine cell lines demonstrated that both star-shaped and linear counterparts have no toxicity at low 10kDa, but significantly toxic at higher 20kDa molar mass, this finding confirmed that the molar mass of PDMAEA play a key role in cytotoxicity effect, not variable polymer structures. Taken together, star-shaped PDMAEA binds more effectively to DNA than linear counterparts and showed no toxicity at 10kDa molar mass at variable polymer concentrations.

Keywords: cationic polymer, gene delivery, star-shaped polymer, cytotoxicity, polyplex

## 1. Introduction

There is a growing evidence that although viral gene delivery systems are the most efficient transfecting agent, their use often induce sever toxicity and immunogenicity [1, 2]. This has led to a surge in use and development of non-viral polycation-based synthetic gene delivery vectors as alternatives to achieve high transfection efficiency with less toxicity, both *in vitro* and *in vivo*[3, 4]. Among which, branched and linear poly (ethylene imine) (PEI) are the most extensively studied polycations used in transfection assay[5]. PEI with a wide range of primary, secondary and tertiary amines groups in its structure which can protonate to efficiently bind to DNA to form into a condense toroidal and globular nanostructures[6], known as polyplex. The PEI/DNA polyplex can then be internalised into cells by endocytosis mechanism[4]. And, it is generally postulated that following internalisation process, the PEI destabilise the endosomal membrane through its buffering capacity ($pK_a$) to result in membrane rupture which then leads to escaping of the polyplex into cytoplasm. However, PEI is also associated with severe toxicity and some studies suggested that the mechanism by which PEI induce toxicity is related to its ability to cause disruption of cell membrane and its integrity followed by activation of mitochondrial mediated apoptosis program[7-9]. These unwanted side effects have spurred the design and engineering of new polycation-based gene delivery systems having different structures and compositions.

Poly(dimethylaminoethyl acrylate) (PDMAEA) has emerged as potential substitute for PEI. This cationic polymer has a buffering capacity higher than that of PEI ($pK_a$ = 7.8) and demonstrates a relatively lower cytotoxicity in cultured cells line[10]. Importantly, its synthesis from the *N, N*-dimethylaminoethyl acrylate (DMAEA), using advanced controlled/living polymerisation techniques, is relatively straightforward [11, 12]. And, numerous PDMAEA polymers with different structure have been reported in literature including linear, branched and dendritic, as transfecting agents. Recently, Georgiou et al

have discussed the role of star polymers in gene delivery and their structure-activity relationship, in which the effect of molecular mass on transfection efficiency and cell viability was investigated [13]. Nonetheless, little attention has been drawn to effect of different polymer architecture of similar molar mass of PDMAEA on binding efficiency to polyanionic DNA, toxicity in culture cell lines and transfection efficiency. More recently, studies have suggested that branched PDMAEA performs better than their linear counterparts as transfecting agent, which attributed to the ability of the branched PDMAEA to densely compact DNA, maintained buffering capacity and lower cytotoxicity[14]. Among the branched PDMAEA, star-shaped polymers are most attractive due to their simplest structure and easy of synthesis and scale-up. Kyriaki *et al*, have studied the transfection efficiency of star-shaped PDMAEA synthesised by "arm-first" approach using Group Transfer Polymerisation (GTP) technique, to delivery siRNA into cultured murine myoblast cells[14]. They reported that the knock-down efficiency increased with polymer loading and branch sizes. In another study, Songrui *et al*, have reported that transfection efficiency of branched PDMAEA increases with increasing degree of branching[15]. Despite these reports, a systematic study that can unravel the effect of PDMAEA architecture and molar mass on binding to polyanionic DNA and study their cytotoxicity in cultured cells line, in directly comparable conditions, is lacking therefore require further investigations.

We report herein the synthesis of a series of new star-shaped (core-first and 4 arms) and linear PDMAEA, in low and high molar mass. We used pentaerythritol tetrakis [2-(dodecylthiocarbonothioylthio)-2-methylpropionate] (4-arm DDMAT) "core-first" and 2-(dodecylthiocarbonothioylthio)-2-methylpropionate Reversible Addition-Fragmentation Chain -transfer (RAFT) agents for the star-shaped and linear PDMAEA respectively. The logic for using these similar chemistry RAFT agents was to maintain a directly comparable synthesis routes and conditions, as well as to control the molar mass of each structure. Furthermore, we aimed to synthesise both 10Ka and 20Ka molar mass to represent a low and high molar mass. The aim was therefore to assess whether the star-shaped PDMAEA would be superior to linear counterpart in both binding efficiencies to DNA and cytotoxicity in cultured cell line.

## 2. Experimental

### *2.1. Materials*

Pentaerythritol tetrakis [2-(dodecylthiocarbonothioylthio)-2-methylpropionate] (4-arm DDMAT, 97%, Sigma), 2- (dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT, 98%, Sigma), 2-butanone (reagent grade, 99% Sigma), chloroform(HPLC grade, Sigma), triethylamine (>99%, Sigma), agarose (bioreagent grade, Sigma), deoxyribonucleic acid (bioreagent, Sigma), ethanol (99%, Sigma), acetone (99%, Sigma), phosphate buffer saline (PBS) tablets (Fisher Chemical), Dulbecco's modified eagle medium (DMEM, Gibco® Fisher), Fetal Calf Serum (FCS, Firstlink), HEPES (bioreagent, 99%, Sigma) and other chemicals were used as received. Gel-Red nucleic acid stain (Biotium US) was purchased from Cambridge Bioscience Limited UK and used as received. 2-(Dimethylamino) ethyl acrylate (DMAEA, 98%, Sigma) was de-inhibited via activated neutral aluminium oxide column before use. 2, 2'-azobis (2-methylpropionitrile) (AIBN, 98%, Sigma) was recrystallized 3 times from acetone prior to use. Pure nitrogen (BOC cylinders) was used in degassing process before polymerisation.

### *2.2. Analytical techniques*

Nuclear magnetic resonance (NMR) spectroscopy. $^{1}H$ and $^{13}C$ NMR spectra were performed using a Bruker 400 MHz spectrometer. Deuterated Chloroform (CDCl3) was used as the solvent for NMR analyses. Data was collected and analysed with TopSpinTM NMR software v.3.2.

Gel permeation chromatography (GPC). Gel permeation chromatography was performed on a G7810A Agilent PL-GPC 50 system connected with refractive index detector. A PLgel 5µm MiniMIX-C (50*4.6mm) guard column was connected to two PLgel 5µm Mixed-D (300*7.5mm) columns connected in series and elevated at a temperature of 30°C. A solution of mixture of HPLC grade chloroform triethylamine (95/5, v/v) was used as the eluent. The machine was calibrated using Agilent EasiVial GPC calibration standards with molar mass ranging from 600 to 300k Da. Polymer solutions (4~5 mg $mL^{-1}$ in the eluent) were injected into the machine through injection valve manually. The flow rate was set at 1 mL $min^{-1}$. Data was collected and analysed with CIRRUSTM data stream software.

Dynamic light scattering (DLS). Dynamic light scattering studies were performed to determine the size of polymers and polyplexes using a Malvern Zetasizer Nano ZS instrument (Malvern, UK) equipped with a maximum 4 mV He-Ne laser operating at 633 nm. The polymer sample solutions were prepared with appropriate buffers at 1 mg·mL-1 concentration. All samples were measured by scanning at least 10 times for each measurement and done in triplicate.

*2.3. Synthesis of linear and star-shaped PDMAEA via RAFT polymerisation*

The polymers were prepared following a modified protocols from literatures [16, 17]. In a typical reaction condition, DMAEA monomer and AIBN initiator were dissolved in 2-butaone and then mixed with either 4 arms – DDMAT RAFT agent (star-shaped polymer) or DDMAT RAFT agent (linear polymer) in a round bottom flask then sealed with a rubber stopper. The individual mixture was then degassed for 30 minutes with nitrogen purging at room temperature with constant stirring, and then the temperature was elevated and maintained at 70°C. Finally, the polymers were recovered by precipitating into *n*-hexane 3x times to remove the unreacted monomers, and then dried under vacuum for 24 hours. Overall two linear (10kDa and 20kDa) and two star-shaped (10kDa and 20kDa) PDMAEA polymers were synthesised and the structures were then analysed by $^{1}$H NMR, $^{13}$C NMR and the molar mass were obtained by GPC using Chloroform-Triethylamine mixture (95/5, v/v) as the eluent, using polystyrene standards. All the reaction conditions and molar ratio of the component are given in **Table 1**. The reaction process was following by the analysis of the chemical structures of both PL and PS polymers using $^{1}$H, $^{13}$C NMR, with the chemical shift at around (δ 4.00(br s, 2H, CH2), referring to the methylene group of the ester bond in the DMAEA monomer was used to monomer conversion during polymerisation. In addition, the reduction in vinyl protons was also monitored as indicator of monomer conversion and polymerisation process. The spectra analysis for PL and PS polymers are given in supporting information (S1-S6). And the extended structural analysis of both PL and PS polymers were recorded as, PL $^{1}$H NMR (400 MHz, CDCl3, δ in ppm): δ 4.00(br s, 2H, CH2), 2.2~2.3(br s, 6H, N(CH3)2), 1.6~1.9(2br s, 2H, CH2), 2.4(br s, 1H, CH backbone), 2.53~2.6(br s, 2H, CH2). $^{13}$C NMR (400 MHz, CDCl3, δ in ppm): δ 174.31 (COOCH2), δ 62.4 (COOCH2CH3), δ 57.6 (COOCH2CH3), δ 45.7 (N (CH3)2), δ 41.9 (C backbone). And for the PS polymers, $^{1}$H NMR (400 MHz, CDCl3, δ in ppm): δ 4.00(br s, 2H, CH2), 2.2~2.3(br s, 6H, N(CH3)2), 1.6~1.9(2br s, 2H, CH2), 2.4(br s, 1H, CH backbone), 2.53~2.6(br s, 2H, CH2). $^{13}$C NMR (400 MHz, CDCl3, δ in ppm): δ 174.31 (COOCH2), δ 62.4 (COOCH2CH3), δ 57.6 (COOCH2CH3), δ 45.7 (N (CH3)2), δ 41.9 (C backbone), were also recorded. The percentage of monomer conversion of all polymers are given **Table.1.**

2.3. Aqueous solution behaviour of linear and star-shaped PDMAEA polymers

To measure the zeta potential, both linear and star-shaped PDMAEA polymers were stirred and dissolved in 10mM HEPES buffer to make a final concentration of 1 mg mL$^{-1}$. And, to measure zeta potential as a function of pH, a series of polymer solutions were prepared with pH of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 values. The samples were analysed with Malvern Nanosizer with Folded Capillary Cells DTS1060 (Malvern Instruments) and data were recorded. All measurements were carried out in triplicates. The hydrodynamic diameter of the linear and star-shaped PDMAEA polymers were also measured as a function of pH, using the same solution condition as above, 15 cycle runs were performed for each sample and the data averaged using the Malvern analysis software. All the samples were made and measured in triplicates.

2.4. Hydrogen ion titration

The protocol of hydrogen Ion titration was adopted and modified from following reference. In short, 50 mg of polymer of polymers (PS10, PS20, PL10 andPL20) were dissolved in 150Mm sodium chloride (NaCl) solution and diluted to a final volume of 10 ml. the pH of all solutions were measured by using Mettler Toledo SevenEasy® pH meter. The polymer solutions were adjusted to pH 2 by using 0.1 M hydrochloride acid (HCl). The solutions were titrated stepwise by adding 0.1 M sodium hydroxide (NaOH) and the pH were measured during the processes. The titration was repeated three times and the mean pH value was reported. The pKa values were calculated from the titration curve.

2.5. Preparation and characterisation of linear and star-shaped PDMAEA/DNA polyplexes

The polyplex solution were prepared accordingly to publish report[18]. A stock solution of all linear and star-shaped PDMAEA polymers were prepared in sterile, nuclease-free water, in 10mM pH 7.4 HEPES buffer, from which a series of dilutions were prepared to obtain a different N/P ratio ranging from 1:1 to 1:15 of polymer to dsDNA. Briefly, dsDNA was dissolved in same buffer solution to prepare a stock solution containing a concentration of 1 µg mL$^{-1}$, and the polyplex formed by mixing an equal volume of the dsDNA solution with the polymer solution in dark, which followed by incubation for 30 minutes.

2.6. Electrophoresis experiment with Agarose Gel

1.5 g of agarose powder was mixed in 100 mL of Tris–acetate– EDTA (TAE) buffer solution. The mixture was heated until the agarose was completely dissolved. To which, 5 µL of GelRed DNA stains (Biotium US) was added as DNA staining. The agarose solution was then carefully poured into the gel tray to allow to cool down to form the agarose gel. Electrophoresis experiments were performed in TAE running buffer. Then, 8 µL of each polyplex solutions were mixed with 2 µL of loading buffer and added into the wells. The experiments were then run at 80V for 40 minutes in a Mini-Sub® cell system (Bio-Rad Laboratories). Bands were visualized and photographed using Gel DocTM XR imaging system and data was analysed with Image LabTM Software (Bio-Rad laboratories).

2.7. Cytotoxicity studies of linear and star-shaped PDMAEA in cultured 3T3 mouse fibroblast cell line

The cytotoxicity studies of linear and 4-arm branched PDMAEA were performed with mice 3T3-fibroblast stem cells in MTS assay. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin (PS) at 37 °C in 5% CO2. 10,000 cells were seeded in 100µL of medium each well in a 96 well plate and incubated for 3 hours for then treated with polymers solutions of different concentration. 24, 48 and 72 hours after polymer treatment, 10µL of MTS was added into each well and incubated for 1hour before reading at a wavelength of 490nm with Omega Plate reader. All the data was analysed and normalized before formatted into figures.

## 3. Results and discussion

3.1. Synthesis of linear and star-shaped PDMAEA using RAFT polymerisation

Linear and star-shaped PDMAEA were synthesised using RAFT polymerisation technique. For the Linear PDMAEA 2- (Dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT) was used as a RAFT agent, whereas pentaerythritol tetrakis [2-(dodecylthiocarbonothioylthio)-2-methylpropionate] (4-arm DDMAT) was used for the star-shaped PDMAEA polymer. The latter macro-RAFT agent has four end-unites of RAFT agent attached to a hydrophobic core structure from which the PDMAEA chains are to grow, thus creating a star-shaped polymer [19, 20], as shown in Fig.1. The logic behind using control/living polymerisation of RAFT for this work was to ensure that control over the molar mass could be achieved which is a critical factor for physicochemical characterisation of all the polymers. And, In order to be able to directly compare the solution behaviour, binding affinity to DNA and cytotoxicity of all linear and star-shaped polymers, of which two are linear and two are star-shaped polymers with theoretical molar masss of 10kDa (representing low molar mass) and 20kDa (representing high molar mass), were prepared and analysed. From here on we refer to the linear and star-shaped polymer as PL and PS respectively, as shown in Table.1

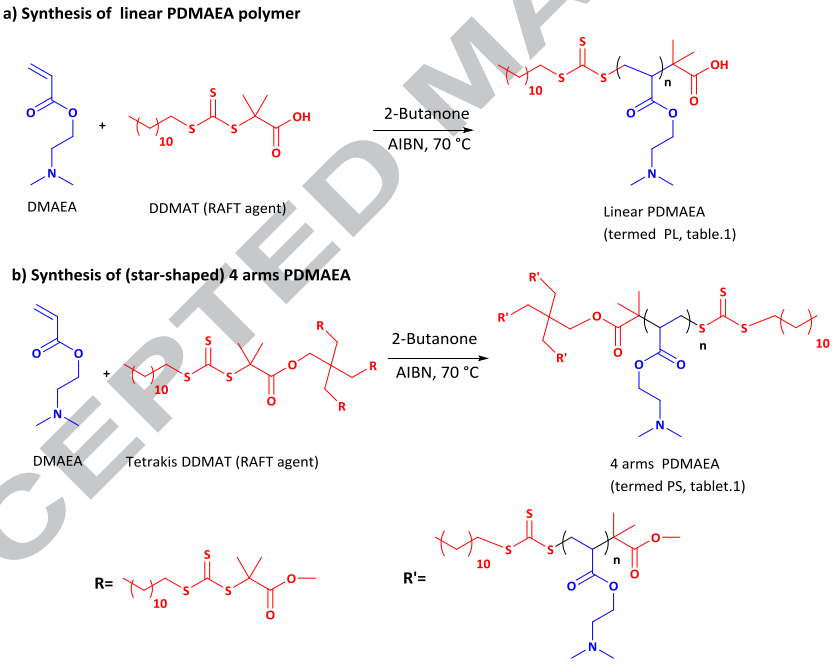



**Fig.1.** Outline the design and synthesis of the linear and star-shaped PDMAEA polymers from DMAEA monomer, using RAFT polymerisation techniques. It should be noted that the DDMAT (RAFT agent) was used to prepare the linear PDMAEA, whereas 4-arm DDMAT analogue was used to prepare the star-shaped PDMAEA. All polymerisation was carried out in 2-Butanone solvent, and at 70 °C in the presence of thermal initiator AIBN.

The molar mass and dispersity of all PL and PS PDMAEA polymers were analysed by GPC, using available polystyrene standard calibration curve (note that the polystyrene standards gives a relative molar mass than the actual values). The results of *Mn*, *Mw* values and PI are given in the **Table 1**. Compared to the theoretical molar mass, the representative value of *Mn* for all the PL and PS polymers show a high degree of agreement between the theoretical and GPC (*Mn* value), suggesting good control over the molar mass was possible using RAFT polymerisation[21, 22]. It must be noted that there is a subtle yet apparent difference in the molar mass evolution as a function of time between the PL and PS polymers. The PS polymers takes slightly longer time to achieve the similar molar mass as the linear ones. This might be due the steric

hindrance imposed by branches of the star shape polymer during chain propagation [23]. Similarly, the Đ(dispersity) value of the PL polymers show a narrower distribution (Đ= 1.15 and 1.14) compared to the PS polymers (Đ= 1.35 and 1.38), the latter is a known characteristics of star – and branched-polymer. The broader Đ value of PS polymers are in agreement with published work[24]. The degree of polymerisation (DP) determined by end group analysis based on $^1$H NMR and the calculated DP for all the polymers are listed in table 1. The DP of PL10 and PL20 were 82.5 and 131.4 respectively. Whereas the DP for the PS10 and PS20 polymers were 105.6 and 187 respectively. This end group analysis suggests a relatively higher DP was achieved the star polymers compared to the linear counterparts.

Table1. Experimental conditions and molar mass characterisation of linear and star-shaped polymers

| Polymer entry | Abbreviation[a] | [Monomer]/[AIBN]/[RAFT][b] Molar ratio | Time(h) | Theoretical Mwt (kDa) | Conv (%)[c] | DP[d] | $M_n$[e] (kDa) | $M_w$ (kDa) | Đ[f] |
|---|---|---|---|---|---|---|---|---|---|
| 10k Linear | PL10 | 100/0.2/1 | 4 | 10 | 66% | 82.5 | 9618 | 11063 | 1.150 |
| 20k Linear | PL20 | 200/0.2/1 | 18 | 10 | 82% | 131.4 | 20996 | 24034 | 1.147 |
| 10k Star | PS10 | 100/0.8/1 | 8 | 20 | 75% | 105.6 | 9354 | 12674 | 1.35 |
| 20k Star | PS20 | 200/0.8/1 | 20 | 20 | 85% | 187 | 19283 | 24529 | 1.382 |

[a]Abbreviation for linear and star-shaped PDMAEA polymers. [b] Molar ratio of monomer: initiator: RAFT agent as was conducted in 2-butanone at 70 °C . [c] Monomer conversion was determined by 1H NMR spectroscopy. [d] Degree of polymerization [e] The number average molar mass and weight average molar mass was determined by GPC using Agilent EasiVial PS standards in triethylamine-chloroform (5-95,v/v).[f] Dispersity

The hydrogen ion titration studies were performed to determine the pKa of the polymers synthesized. From the titration curve the pKa values were calculated and recorded as 7.81, 7.52, 7.80 and 7.79 for PL10, PL20, PS10 and PS20 respectively. This suggest that the pKa values were not significantly affected by the structure.

Next, the aqueous solution behaviour of both PL and PS PDMAEA polymers were investigated using both dynamic light scattering (DLS) and zeta potential measurement (zeta), results are shown in Fig. 2 and discussed in the following section.

First, all the DLS and Zeta measurements in buffer solution with a wide range of pH (HEPES buffer, 10mM) from 6, 6.5, 7, 7.5 and 8, in order to emulate both the extracellular and intracellular pH conditions. Results are shown in Fig. 2a, b, c, d. we have also looked at the analysis by intensity, number and volume distribution. Fig.2a, c represents the hydrodynamic diameters as a function of pH for PL10 and PL20, whereas, Fig. 2c, d represent the hydrodynamic diameters for PS10 and PS20, also as a function of pH value. Except for the PL20 and PS20 at pH 8, all the other polymers showed a narrow distribution of sizes in the ranges of 6-10 nm, at all pH condition. PL20 and PS20 polymers were both showed aggregation at higher pH 8, this is unspringing as the effect of pKa values of DMAEA monomers become more apparent, therefore the deprotonation of the amine groups on the DMAEA monomer backbone could possibly lead to reduction in polymer solubility aqueous condition, thus forcing hydrophobic aggregation to happen. And this was apparent only at higher molar mass polymers. Nonetheless, the overall hydrodynamic diameter of both PL and PS polymers appear to be similar in all pH conditions.

Similarly, the zeta potential measurements were carried out over a wide pH range of 5.5, 6, 6.6, 7, 7.5, 8, 8.5 and 9 values for all PL and PS PDMAEA polymers. Interestingly, at a lower pH value of 6, when the DAMEA monomers are ionised and protonated, the PS polymers showed a subtle different compared to PL polymers. Both PS10 and PS20 recorded a zeta potential measurement of ≥50mV, whereas PL10 and PL20 measurements were around 40mV and 45mV respectively. This phenomenon was less apparent over higher pH ranges. Though this seems insignificant but at a lower pH which is correspondent to endosomal pH the PS PDMAEA polymers might have a better buffering capacity [25, 26]. Importantly, all the PL and PS PDAEMA polymers zeta potential measurements followed a similar trend of reduction in value as the pH increased, and the zeta potential dropped below zero at pH close to the pKa value of DMAEA. However, at the physiological pH 7.5 and lower, all the PL and PS PDMAEA polymers maintained a positive zeta potential value above 20mV, suggesting a colloidal stability achieved and maintained.

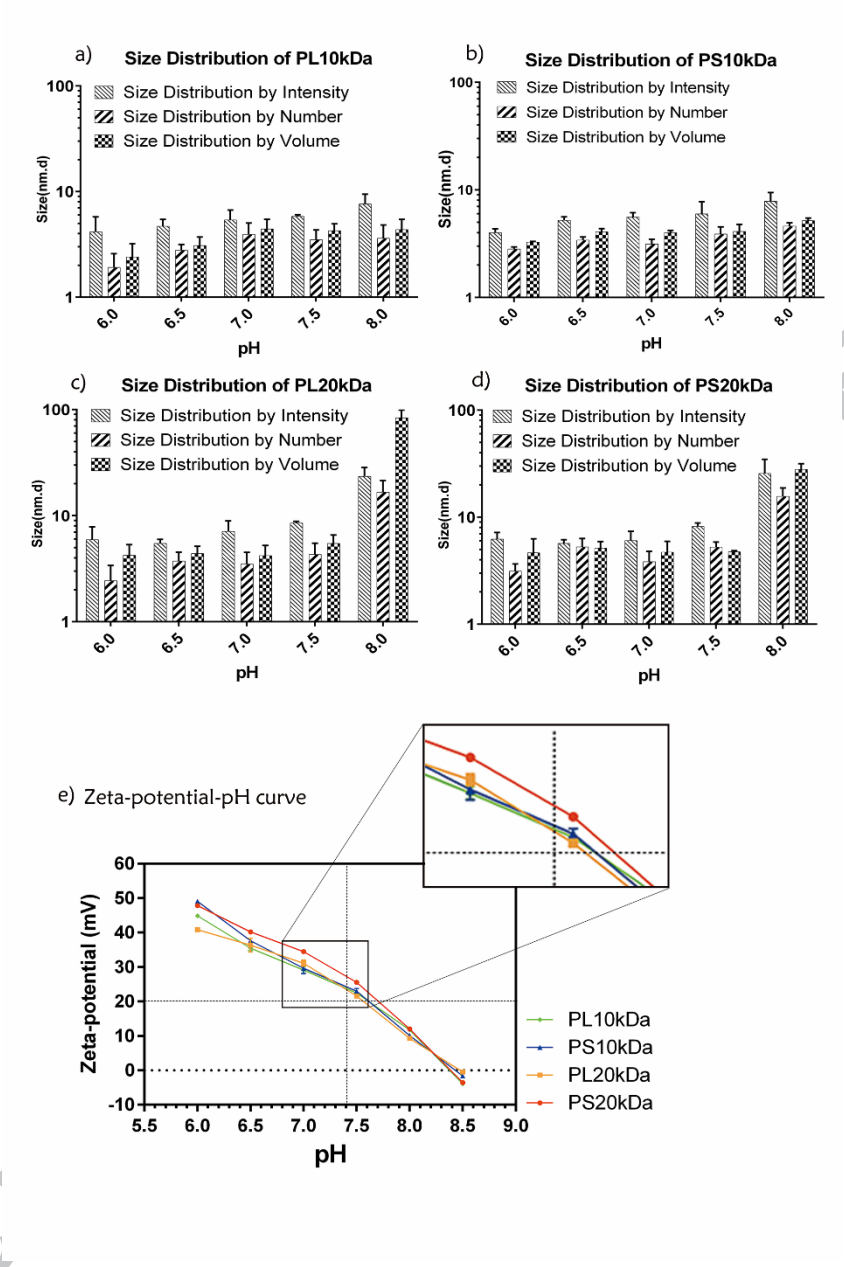



**Fig.2.** Dynamic light scattering and zeta potential measurement as a function of pH. a-d, showing hydrodynamic diameters of a) PL10kDa (linear), b) PS10kDa (star-shaped), c) PL20kDa (linear), and d) PS20kDa (star-shaped) polymers as a function of pH, prepared in HEPES buffer 10mM. And, e) zeta potential diagram as a function of pH for all PL10kDA, PS10kDa, PL20kDa and PS20kDa, measured in HEPES Buffer 10mM. The zoomed in inset of the diagram depicts the zeta potential measurement of all linear and star-shaped polymers at physiological pH, values are higher than 20mV indicating good colloidal stability.

Next, the binding affinity of the PL and PS PDMAEA polymers to DNA as a function structure (linear vs. star-shaped) and molar mass (10kDa and 20kDa) were investigated and using agarose electrophoresis gel analysis. The agarose gels were prepared following published protocols. A short double stranded DNA (dsDNA) were used for polyplex formation, as a model macromolecules, as opposed to circular DNA since our research interest is to design and engineer a delivery system for therapeutic RNA (which often available in short double strand base pair). The agarose electrophoresis were performed for all PL10kDa, PL20kDa, PS10kDa and PS20kDa to investigate both the effect of structure and molar mass on binding to dsDNA. In all experiments, naked dsDNA was used as control. A series of nitrogen to phosphate ratio (N/P) were investigated in different lanes ranging from 1-15 (DNA: polymer ratio), i.e. increasing the amount of polymer but maintaining the DNA ratio at 1 in all cases. As shown in Fig.3, the disappearance of DNA band at the bottom of the gel at certain N/P ratio were chosen as a ratio at which the polyplex formulation is strong and complete (free from unbound DNA). Interestingly, the results show a trend between PL (linear) and PS (star-shaped) PDMAEA polymers, which demonstrate that the PS10kDa (a) and PS20kDa (c) bind to DNA at a lower N/P ratio (1:5 and 1:3 respectively), whereas the PL10kDa (b) and PL20kDa (d) polymers bind to

DNA at a higher N/P ratio (1:9 and 1:5 respectively. This apparent differences in binding affinity between linear and star-shaped polymers might be due to the fact that the star-shaped polymer are rigid 3D structures and more accessible than linear counterparts. Similar finding has been reported indicating our work is in agreement with published work [27]. In addition, unsurprisingly, with all polymers at the molar mass increases, the N/P ratio decreases, suggesting that more and more nitrogen groups are available in higher molar mass polymers.

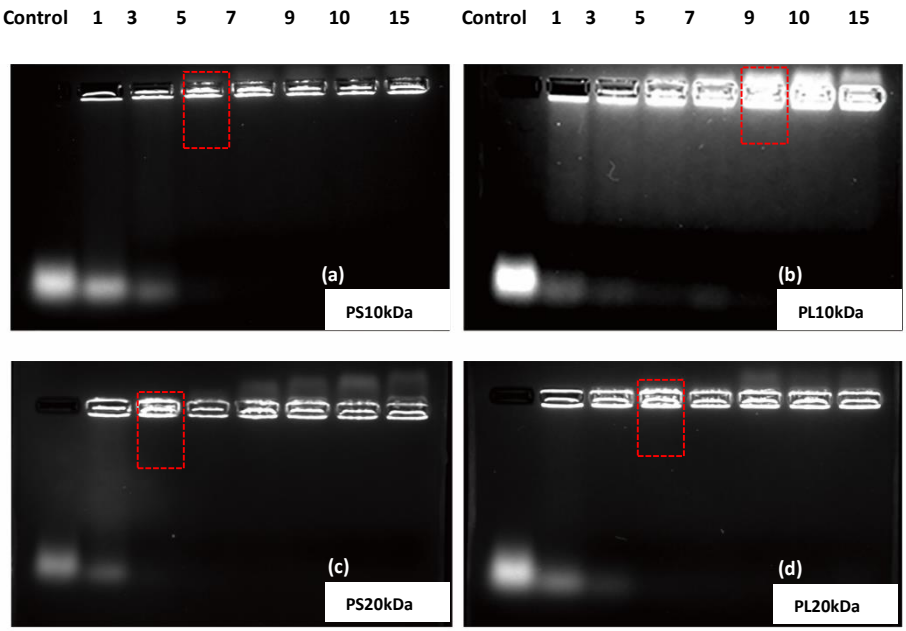
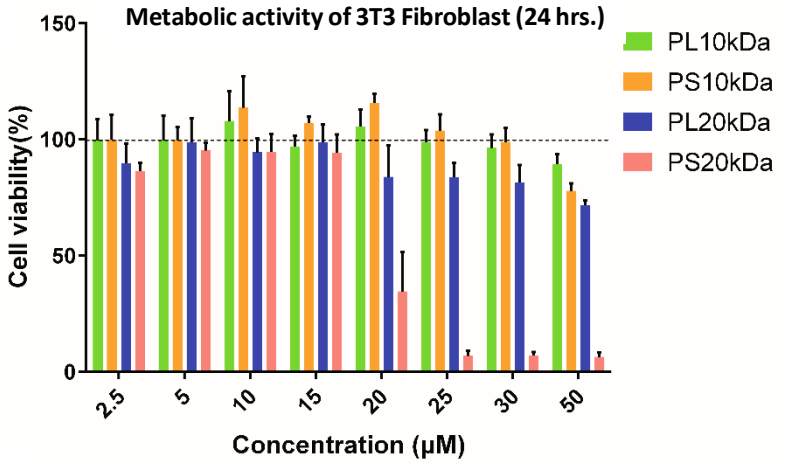



**Fig.3.** Electrophoresis analysis to investigate the effect of structure and molar mass on binding affinity of the PL (linear) and PS (star-shaped) PDMAEA structure to DNA. Control in lane 0 indicate naked DNA, and numbers are correspondence to N/P ratio. a) The PS10kDa (star-shaped) formed complex at N/P ratio 5:1, b) PL10kDa (linear) formed complex at N/P ratio 9:1, c) PS20kDa (star-shaped) formed complex at N/P 3:1, and d) PL20kDa formed complex at N/P ratio 5:1. The disappearance of DNA band at the bottom of the gel was chosen as indicator for polyplex formation compared to the control. The agarose gel was performed in TAE buffer at 80mV for 40 minutes. Agarose concentration used was 1.5% and GELRED® as DNA gel stain.

Finally, the effect of structure and molar mass of PL (linear) and PS (star-shaped) PDMAEA polymers on metabolic activity of 3T3 fibroblast cultured cell line were investigated. MTS assay, which is routinely used to measure the cytotoxicity of polymers in cultured cell lines, was used in this work at different time intervals of 24, 48 and 72 hours. And, to study the effect of concentration of both the PL and PS polymers, a series of concentration from 2.5 to 50 µM (prepared in HEPES Buffer 10mM) (covering the N/P ratio ranges used in Fig.3) were investigated. Most importantly, the effect of structure and molar mass was studied using PL10kDa, PL20kDa (linear polymers) and PS10kDa and PS20kDa (star-shaped) polymers, as shown in Fig.4. Noticeably, both the PL10kDa and PS10kDa showed no or minimal (less than 10%) cytotoxicity effect at all concentrations and over 24, 48 and 72 hrs timeframes, when compared and normalised to a control of cell plus media only. In contrast, both PL20kDa and PS20kDa showed considerable toxicity (more than 20%) at concentrations of 2.5 – 10 µM and significant toxicity (more than 50%) at concentrations of 15 -50 µM, at all interval timeframes.

Taken together, these results indicated that indeed the molar mass of the PDMAEA has the sole effect on cytotoxicity, and that the PDMAEA structures of linear and star-shaped produced no difference on the toxicity. Nonetheless, the polymers PL and PS at low molar mass of 10kDa seem to be a good candidate to take it forward as transfecting agent, with the PS polymers shows better binding to DNA than the PL counterparts.

a)

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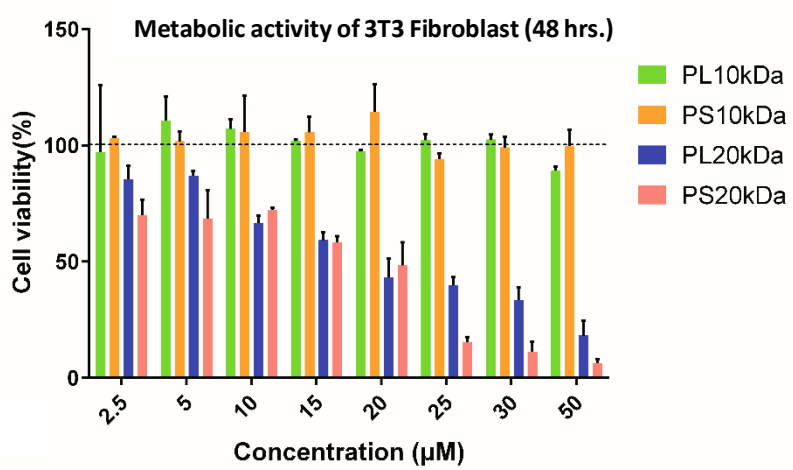


c)

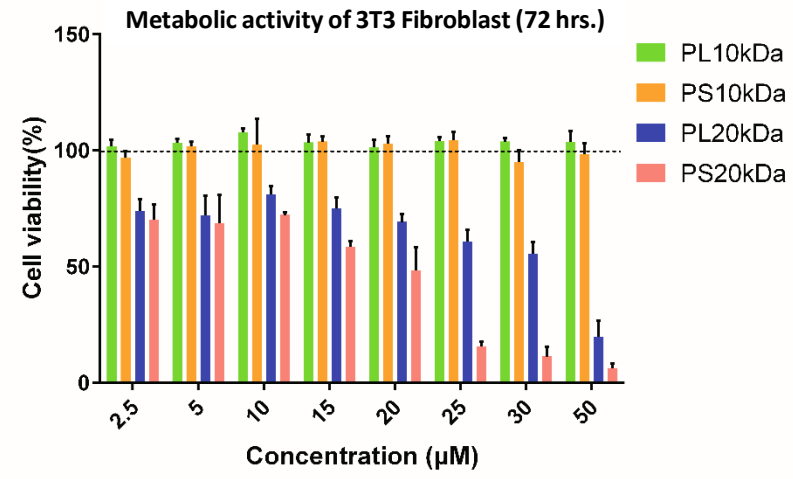


**Fig.4.** Cell metabolic activities as a marker of cell viability of 3T3 fibroblast cell as evaluated by the MTS assay following incubation with PL10kDa, PS10kDa, PL20kDa and PS20kDa polymers, at concentration of 2.5 to 50 µM polymers. And over a) 24 hrs, b) 48 hrs and c) 72 hrs time intervals.

## 4. Conclusions

Linear and star-shaped PDMAEA polymers were synthesised via RAFT polymerisation in two molar mass format of 10kDa and 20kDa, and directly compared their aqueous solution behaviour, binding affinity to DNA and cytotoxicity effect in 3T3 fibroblast cultured cell line. Noticeably, both linear and star-shaped PDMAEA showed similar hydrodynamic diameter when analysed as a function of pH, with both polymers seemed to aggregate near the pKa of the DMAEA side-chain, perhaps due to reduction in polymer solubility at higher pH value. And, both linear and star-shaped polymer, at both molar mass, showed a pH dependent zeta potential measurement as was expected, with star-polymers seemed to generate slightly higher overall positive charge. However, surprisingly the star shaped polymers (PS), at both 10kDa and 20kDa showed significantly better binding to DNA comparing to linear counterparts, when tested by agarose electrophoresis assay. Nonetheless, we did not find any effect of polymer structure on cytotoxicity when tested both linear and star-shaped polymers in 3T3 cultured cell lines, but cytotoxicity effect was found to be solely affect by molar mass of the polymers. These finding should provide a valuable insight for a direct comparison of linear and star-shaped PDMAEA based polymers for application as gene delivery vector.

**Supporting information.** $^{1}H$, $^{13}CNMR$, GPC chromatograph of the polymers are provided in supporting information document.

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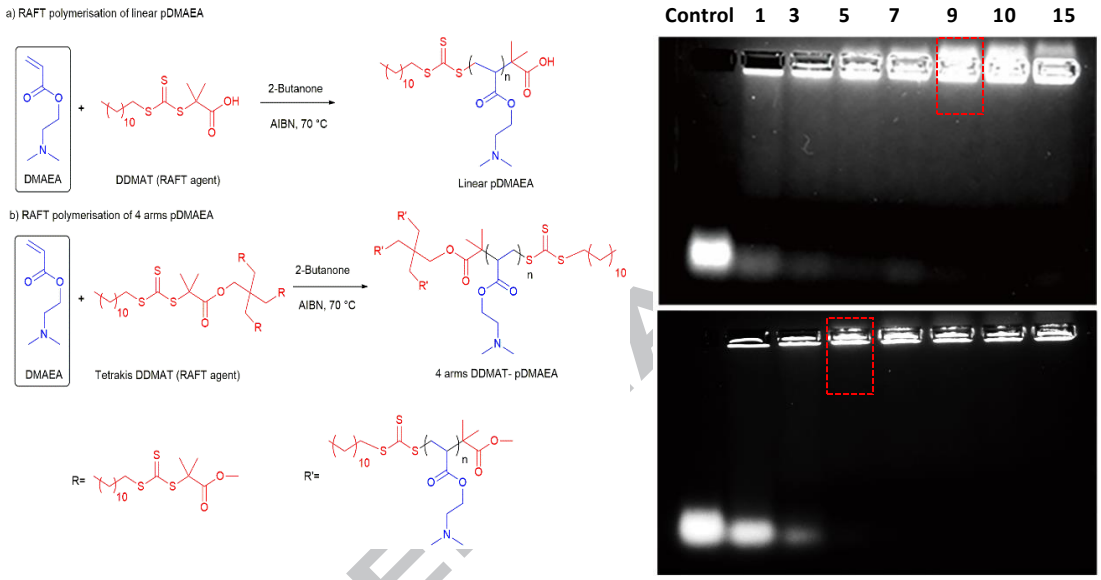
# A direct comparison of linear and star-shaped poly(dimethylaminoethyl acrylate) polymers for polyplexation with DNA and cytotoxicity in cultured cell lines

Xin Liao[a], Grace Walden[a], Noelia D Falcon[a], Simon Donell[b], Mike Raxworthy[c], Michael Wormstone[d], Graham P Riley[d] and Aram Saeed[a]*



# A direct comparison of linear and star-shaped poly(dimethylaminoethyl acrylate) polymers for polyplexation with DNA and cytotoxicity in cultured cell lines

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**Highlights:**

- Effect of linear and star-shaped structure of cationic polymers on DNA binding and cytotoxicity investigated
- Star-shaped polymer form polyplex at smaller nitrogen/phosphate ratio than linear counterparts, indicates effective binding ability
- Molecular weight of the cationic polymers is determinant of cytotoxicity, insignificant toxicity was observed both linear and star-shaped structure at low (10kDa) molecular weight, but showed highly toxic at higher molecular weight (20kDa)